Biological and Molecular Characteristics of *Mycobacterium tuberculosis* Clinical Isolates with Low-Level Resistance to Isoniazid in Japan[▽]

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We reevaluated the BACTEC MGIT 960 antimicrobial susceptibility testing system (MGIT 960 AST) by using 1,112 isolates of *Mycobacterium tuberculosis*. When the results of MGIT 960 AST were compared with that of the proportion method using Ogawa medium (Ogawa PM), discrepant results were obtained for 30 strains with isoniazid, all resistant by MGIT 960 AST but susceptible by Ogawa PM. For 93% of the strains that produced discrepant results, the MIC was 0.4 or 0.8 μg/ml, showing resistance by the proportion method using Middlebrook agar plates. Furthermore, it was also established by analyses of the *katG* and *inhA* genes that strains resistant only by MGIT 960 AST have a low level of isoniazid (INH) resistance, indicating that MGIT 960 AST is a reliable method. Ninety-six strains were resistant to 0.1 μg/ml INH by MGIT 960 AST. When they were divided into three groups, Low-S (susceptible at 0.2 μg/ml), Low-R (resistant at 0.2 μg/ml), and High-R (resistant at 1.0 μg/ml), by Ogawa PM, 43.3% of the Low-S strains had mutations in the promoter region of *inhA* and no mutations were detected in *katG* codon 315, while 61.7% of the High-R strains had *katG* codon 315 mutations or a gross deletion of *katG*. These results suggest that mutations in *inhA* are associated with low-level resistance to INH and *katG* codon 315 mutations are associated with high-level resistance to INH. In addition, the analyses demonstrated some relationship of mutations in the *inhA* gene with ethionamide resistance for the Low-S strains, but not for the High-R strains.

The number of multidrug-resistant strains of *Mycobacterium tuberculosis*, defined as resistant to at least isoniazid (INH) and rifampin (RFP), has been increasing over the years, and several outbreaks have been reported (5, 9, 14, 35, 41, 42). The development of resistance to these drugs reduces the efficacy of standard antituberculosis treatment. It is very important, therefore, to identify these strains as soon as possible to allow for adjustments in treatment and to minimize the transmission of drug-resistant strains.

Phenotypic drug susceptibility testing by conventional methods on solid media (Middlebrook agar, Löwenstein-Jensen egg, and Ogawa egg medium) requires 3 to 4 weeks after the primary culture has been isolated. This time can be reduced by the use of a rapid method such as the BACTEC 460 TB system, which requires 5 to 10 days (32). In some countries, including Japan, however, the use of the BACTEC 460 TB system is restricted because it is difficult to discard the used medium bottles with radioactive substances. In Japan, therefore, drug susceptibility testing of *M. tuberculosis* is now routinely done by the proportion method using Ogawa egg medium (Ogawa PM)

in which critical concentrations comparable to the conventional proportion method using Löwenstein-Jensen medium have been established (13).

Recently, a nonradiometric BACTEC MGIT 960 antimicrobial susceptibility testing system (MGIT 960 AST) has been developed to allow susceptibility testing of *M. tuberculosis* for INH, RFP, streptomycin (SM), ethambutol, and pyrazinamide. We have previously evaluated the MGIT 960 AST for introduction into routine laboratory work (1, 4, 23). During our evaluation of the MGIT 960 system, we found some results that were discrepant by MGIT 960 AST and Ogawa PM in the testing of susceptibility to INH. All of the strains that produced discrepant results showed a phenotype of resistance to INH by MGIT 960 AST but susceptibility by Ogawa PM. Therefore, we reevaluated the MGIT 960 AST by using a large number of clinical isolates of *M. tuberculosis* (28).

In this study, we examined some biological, biochemical, and molecular properties of the strains with discrepant results and compared them with the strains that were resistant by both MGIT 960 AST and Ogawa PM.

MATERIALS AND METHODS

Clinical isolates. A total of 1,112 *M. tuberculosis* strains isolated from different patients from various districts of Japan (12 hospitals from northern Hokkaido to southern Kyushu districts) were studied. All of the isolates were differentiated by an immunochromatographic assay (2) (Capilia TB; Nippon Becton Dickinson

Co. Ltd., Tokyo, Japan) and an RNA-DNA hybridization assay (Gen-Probe, San Diego, CA) by using commercial kits for culture confirmation and identification of species belonging to the *M. tuberculosis* complex, as well as conventional biologic and biochemical tests.

Susceptibility testing by MGIT 960 AST and Ogawa PM. Test organisms were grown in Middlebrook 7H9 liquid medium at 37°C until the optical density at 540 nm reached about 0.2. Then, for the inoculum, the cultures were adjusted to a 0.5 McFarland standard. Testing of susceptibility to INH was performed with an automated MGIT 960 AST (at a critical concentration of 0.1 μg/ml) and by the proportion method using Ogawa egg slant medium (at concentrations of 0.2 and 1.0 μg/ml), which is similar to Löwenstein-Jensen medium. For the Ogawa PM, the strains were considered to be resistant if >1% of the test bacterial population grew at an INH concentration of 0.2 μg/ml.

INH susceptibility testing by MIC determination. The MIC of INH was determined with Middlebrook 7H10 agar medium containing twofold concentrations of the drug ranging from 0.05 to 0.8 μg/ml. MIC testing was performed only with the strains that yielded discrepant results by the two methods.

ETH susceptibility testing. Susceptibility to ethionamide (ETH) was tested by the proportion method using Middlebrook 7H10 agar medium (at a critical concentration of 5.0 μg/ml). Only the resistant isolates were subjected to ETH MIC testing with Middlebrook 7H10 agar.

Catalase activity. Catalase activity was assayed according to Kubica et al. (24). Briefly, strains were cultivated in Middlebrook 7H9 broth. One hundred microliters of the bacterial suspension was inoculated onto an Ogawa egg deep with a horizontal surface in screw-cap tubes and incubated at 37°C for 2 weeks. One milliliter of freshly prepared Tween 80-hydrogen peroxide reagent was then added, and the mixture was incubated at room temperature for 5 min. The height (in millimeters) of the bubbles that formed was then measured. Testing was performed only with the strains that yielded susceptibility results that were discrepant between the two methods.

Sequencing of *katG* and *inhA*. The oligonucleotide primers used were designed to amplify a 1,073-bp fragment of *katG* encompassing codon 315, a 217-bp fragment containing the promoter of *mabA-inhA*, and a 460-bp fragment of the *inhA* open reading frame (ORF). A small amount of numerous colonies from Ogawa egg slants was suspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in a screw-cap tube and then heated at 95°C for 30 min in a water bath. Five microliters of the heat-treated samples was used for amplification in a total reaction volume of 50 μl. Sequencing reactions were performed with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems Inc., Foster City, CA). The products were sequenced with an Applied Biosystems 3130x1 Genetic Analyzer.

Nucleotide sequence accession numbers. The GenBank accession numbers are X68081 for the *katG* gene and U41388 for the *inhA* gene.

RESULTS

A total of 1,112 *M. tuberculosis* strains were tested by MGIT 960 AST and Ogawa PM. Of the isolates tested, 96 (8.6%) and 66 (5.9%) were found resistant to INH by MGIT 960 AST and Ogawa PM, respectively. Thirty strains showed results that were discrepant between the two methods. All of these strains were resistant to INH by MGIT 960 AST and susceptible by Ogawa PM, and no strains were susceptible by MGIT 960 AST and resistant by Ogawa PM.

MICs of INH determined with Middlebrook 7H10 agar medium showed that for 28 out of 30 strains, the MIC was 0.4 or 0.8 μg/ml; for 9 isolates, the MIC was 0.4 μg/ml; and for 19 isolates, the MIC was 0.8 μg/ml (Table 1). The remaining two strains, no. 13 and 29, showed an INH-susceptible phenotype (MIC, 0.2 μg/ml).

Previous studies have shown that strains with high-level resistance to INH completely lost catalase activity (11, 12, 15). We determined if these strains with discrepant results displayed catalase activity. The semiquantitative catalase test revealed that all of the strains had catalase activity, although there were some differences in the amounts of catalase produced (Table 1).

To further confirm the susceptibility of the strains with dis-

crepant results, we examined the genes involved in resistance to INH, *katG* and *inhA*, for mutations. Mutations were found in 15 (50.0%) out of the 30 strains that produced discrepant results (Table 1). One strain had mutations in two genes, and the remaining 14 had a mutation in a single gene. Thirteen strains (43.3%) carried a single point mutation, an *inhA* regulatory region −15 C→T nucleotide substitution. No mutations were seen in the *inhA* ORF of the strains that produced discrepant results. Though resistance-associated mutations in the *katG* gene were identified in three strains (Trp-204-Arg, Gly-285-Asp, and Trp-505-Ser), there were no strains with a mutation at codon 315. Two strains for which the MIC was 0.2 μg/ml had no mutations in the regions of the two genes analyzed.

On the other hand, we also investigated the *katG* and *inhA* genes of 66 strains with a resistance phenotype by both MGIT 960 AST and Ogawa PM. The results are shown in Table 2. Of 66 INH-resistant strains, 50 (75.8%) had mutations in these genes. Resistance-associated mutations within the *katG* gene were found in 38 strains (57.6%). Twenty-seven strains (41.0%) had mutations at *katG* codon 315. The wild-type codon, AGC (Ser), was altered to ACC (Thr) in 24 strains and to AAC (Asn) in 3 strains. Other resistance-associated mutations in the *katG* gene were identified at codons Trp-198-stop, Glu-217-Gly, Gly-285-Cys, Tyr-337-Cys, and Thr-344-Pro. No amplification of the *katG* gene was seen in five strains. Analyses of the 5′ end of a presumed ribosome-binding site in the promoter of *mabA-inhA* revealed nucleotide substitutions of C→T at −15 in nine strains and G→T at −17 in three strains. The *inhA* ORF mutation (Ser-94-Arg) occurred in three strains. All three strains had additional mutations in *katG* or the promoter of *inhA*.

Previous findings indicated that the *katG* codon 463 mutation was not associated with INH resistance (19, 39). In our study, 83.3% (25/30) of the strains with results that were discrepant between the two methods and 71.2% (47/66) of the strains found resistant by both methods carried a mutation in *katG* codon 463 (Arg→Leu).

Ninety-six strains were resistant to INH at 0.1 μg/ml by MGIT 960 AST. They were divided into three groups based on the results obtained with the Ogawa PM, i.e., (i) Low-S (susceptible at 0.2 μg/ml), (ii) Low-R (resistant at 0.2 μg/ml and susceptible at 1.0 μg/ml), and (iii) High-R (resistant at 1.0 μg/ml). They included 30, 19, and 47 strains, respectively. Of 47 High-R strains, 24 (51.1%) carried mutations in *katG* codon 315 and 5 (5.2%) had a gross *katG* deletion (Table 3). On the other hand, no mutations were detected in *katG* codon 315 of any of the Low-S strains. Thirteen strains (43.3% [13/30]) of the Low-S group and 10 strains (52.6% [10/19]) of the Low-R group had mutations in the promoter of *inhA*, while only 2 strains (4.3% [2/47]) of the High-R group had a mutation in this region.

As shown in Table 4, 43 of 96 INH-resistant strains were also resistant to ETH by the proportion method using Middlebrook 7H10 agar. When, as before, they were divided into three groups based on the results of INH susceptibility testing with the Ogawa PM (11 strains in the Low-S group, 15 in the Low-R group, and 17 in the High-R group), 100% of the Low-S, 66.7% of the Low-R, and 5.9% of the High-R strains had mutations in the promoter of *inhA* or in the *inhA* ORF. How-

TABLE 1. Some characteristics of *M. tuberculosis* isolates with INH susceptibility test results discrepant between MGIT 960 AST and Ogawa PM

Isolate	Susceptibility to INH[a]		MIC[b] (µg/ml)	Catalase activity[c] (mm)	Mutation in:		Resistance[e]
	MGIT 960 AST	Ogawa PM			*katG*[d]	*inhA* promoter	
1	R	S	0.8	1			SM
2	R	S	0.8	10			
3	R	S	0.4	9			
4	R	S	0.8	7			
5	R	S	0.4	13			SM
6	R	S	0.8	13			RFP, EMB
7	R	S	0.8	14		−15 C→T	ETH
8	R	S	0.8	24		−15 C→T	SM, ETH
9	R	S	0.8	24		−15 C→T	
10	R	S	0.8	2	Gly285Asp		
11	R	S	0.8	12			
12	R	S	0.4	10			SM
13	R	S	0.2	11			SM
14	R	S	0.4	22			
15	R	S	0.8	10		−15 C→T	SM, ETH
16	R	S	0.8	4	Trp505Ser		
17	R	S	0.8	2	Trp204Arg	−15 C→T	KM, ETH
18	R	S	0.4	8		−15 C→T	KM, ETH
19	R	S	0.8	12			
20	R	S	0.8	16			
21	R	S	0.8	15		−15 C→T	SM, ETH
22	R	S	0.8	22			
23	R	S	0.8	9		−15 C→T	ETH
24	R	S	0.8	18		−15 C→T	ETH, FQ
25	R	S	0.4	11		−15 C→T	ETH
26	R	S	0.4	33		−15 C→T	SM, ETH
27	R	S	0.4	16		−15 C→T	SM, ETH
28	R	S	0.4	18		−15 C→T	SM, ETH
29	R	S	0.2	8			
30	R	S	0.8	4			

[a] Abbreviations: R, resistant; S, susceptible.
[b] MICs of INH were determined with Middlebrook 7H10 agar medium containing twofold concentrations of the drug ranging from 0.05 to 0.8 µg/ml.
[c] Catalase activity was assayed according to Kubica et al. (22). The height (in millimeters) of the bubbles was measured.
[d] Excluding the *katG* Arg-463-Leu polymorphism.
[e] Abbreviations: RFP, rifampin; EMB; ethambutol; KM, kanamycin; FQ, fluoroquinolone.

ever, no relationship was seen between differences in the distribution of MICs of ETH and mutations in the *inhA* gene (data not shown).

DISCUSSION

Many studies have demonstrated that MGIT 960 AST is a reliable method for testing the drug susceptibility of *M. tuberculosis* (1, 4, 8, 33, 34, 37). Some studies, however, have shown discrepant results between MGIT 960 AST and other methods such as BACTEC 460 TB or Ogawa PM in the testing of susceptibility to INH (1, 8, 28, 37). INH is an important first-line antituberculosis drug that, along with RFP and pyrazinamide, forms the basis of the widely used directly observed treatment short course for tuberculosis (43). Therefore, we reevaluated the MGIT 960 AST by using 1,112 clinical isolates of *M. tuberculosis*. When the results of the MGIT 960 AST were compared with those of the Ogawa PM, INH results for 30 strains that were discrepant between the two methods were found (resistant by MGIT 960 AST and susceptible by Ogawa PM). For 28 (93.3%) out of 30 strains that produced discrepant results, the MIC was 0.4 or 0.8 µg/ml, showing resistance by testing at 0.2 µg/ml with the proportion method using Middlebrook 7H10 agar, which is recommended by the Clinical and

Laboratory Standards Institute of the United States (10). The results were also confirmed by analysis of the *katG* and *inhA* genes involved in resistance to INH. These data indicate that the fully automated and nonradiometric MGIT 960 AST is an accurate method for rapid susceptibility testing of *M. tuberculosis*.

Our present study shows that strains with low-level INH resistance may show resistance by MGIT 960 AST while they are susceptible to INH by Ogawa PM, and probably also by the proportion method using Löwenstein-Jensen medium. In this study, 11 out of 30 discrepant cases were followed up clinically and no relapses were identified. Since multiple-drug therapy is used, it is not clear if low-level INH resistance plays any significant role in the development of INH resistance in such patients. On the other hand, INH is used for the treatment of patients with latent tuberculosis infection (3). Whether INH is effective in the treatment of such patients remains unsolved.

In our study of 96 INH-resistant isolates in Japan, 65 (67.7%) had a mutation in the structure of *katG* or *inhA* and/or in the promoter of *inhA* and 31 (32.3%) had no mutations in the fragments analyzed. Among these strains, mutations in the *katG* gene were predominant (42.7% [41/96]). We observed that only 28.1% (27/96) of the strains resistant to INH carried a *katG* codon 315 mutation, which is contrary to results in

TABLE 2. Mutations in the *katG* and *inhA* genes of *M. tuberculosis* strains resistant to INH by both MGIT 960 AST and Ogawa PM

Gene(s)	Codon	Nucleotide change	Amino acid substitution	No. of isolates
katG[a]	198	TGG→TGA	Trp→Stop	1
	217	GAG→GGG	Glu→Gly	1
	285	GGC→TGC	Gly→Cys	1
	315	AGC→ACC	Ser→Thr	24
	315	AGC→AAC	Ser→Asn	3
	337	TAC→TGC	Tyr→Cys	1
	344	ACG→CCG	Thr→Pro	1
katG (no amplification)				5
inhA promoter	−15	C→T		9
	−17	G→T		1
inhA promoter + *inhA* ORF	−17	G→T		2
	94	TCG→GCG	Ser→Ala	
katG + *inhA* ORF	217	GAG→GGG	Glu→Gly	1
	94	TCG→GCG	Ser→Ala	
katG + *inhA*		None	None	16

[a] Excluding the *katG* Arg-463-Leu polymorphism.

northwestern Russia (93.6%), in Latvia (91.0%), in Lithuania (85.7%), in Germany (88.4%), and in China (64.4%) (6, 20, 29, 38, 44). In comparison with the low frequency of this mutation in Japanese strains, it was found in 46% of the strains tested in Madrid and 37.8% of the strains tested in Italy (16, 31). Looking at our data and the low frequency of the *katG* codon 315 mutation, this indicates that the proportion method using Löwenstein-Jensen or Ogawa medium may fail to detect some strains with low-level resistance to INH, compared with MGIT 960 AST. Indeed, when the frequency is analyzed on the basis of the susceptibility results obtained with Ogawa PM, it increases from 28.1% (27/96) to 40.9% (27/66).

Since 57.3% (55/96) of our INH-resistant strains had no mutation in the *katG* gene (Tables 1 and 2), it is likely that mutations in other genes, such as the *inhA* locus, significantly contribute to resistance (7). Previous studies have shown that mutations in the upstream region of the *inhA* locus result in increased levels of InhA (NADH-dependent enoyl-acyl carrier protein reductase) expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism (30). Our search for mutations in the *inhA* regulatory region of INH-resistant *M. tuberculosis* strains found a substitution at position 15 upstream of the start codon in 22 (22.9%) strains and at position 17 in 3 (3.1%) strains. This mutation frequency is comparable to those in previous reports, in which the mutations were found in 10 to 34.2% of the INH-resistant isolates tested (17, 21, 22, 26, 36).

M. tuberculosis strains that were INH resistant by MGIT 960 AST were divided into three groups (Low-S, Low-R, and High-R) based on the results of INH susceptibility testing with the Ogawa PM. Around 50% of the High-R strains had *katG* codon 315 mutations, and only 4.3% (2/47) carried a mutation in the promoter region of *inhA* (Table 3). On the other hand, 43.3% (13/30) of the Low-S strains had a mutation in the promoter of the *inhA* gene and no mutation was seen in *katG* codon 315 of these strains. Low-R strains showed a mutation frequency intermediate between those of the Low-S and High-R strains. These results indicate that *katG* codon 315 mutations are associated with high-level resistance to INH and that *inhA* regulatory region mutations are associated with low-level resistance to INH (only 4.3% of the High-R strains had mutations in the promoter of *inhA*), supporting previous re-

TABLE 3. Relationships between levels of resistance to INH and mutations in *katG* codon 315 or in the promoter of *inhA*

Susceptibility to INH determined by Ogawa PM	No. of strains[a]	No. (%) of strains with mutations in:	
		katG codon 315	*inhA* promoter
Low-S[b]	30	0	13 (43.3)
Low-R[c]	19	3 (15.8)	10 (52.6)
High-R[d]	47	24 (51.1)	2 (4.3)

[a] All strains were resistant to INH at 0.1 μg/ml by MGIT 960 AST.
[b] Low-S, susceptible to INH at 0.2 μg/ml by Ogawa PM.
[c] Low-R, resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml by Ogawa PM.
[d] High-R, resistant to INH at 1.0 μg/ml by Ogawa PM.

TABLE 4. Mutations in the *inhA* gene of *M. tuberculosis* isolates with resistance to both INH and ETH

Susceptibility to INH determined by Ogawa PM	No. of strains[a]	No. (%) of strains with mutations
Low-S[b]	11	11 (100)
Low-R[c]	15	10 (66.7)
High-R[d]	17	1 (5.9)

[a] All strains were resistant to INH at 0.1 μg/ml by MGIT 960 AST and to ETH at 5 μg/ml by the proportion method using Middlebrook 7H10 agar.
[b] Low-S, susceptible to INH at 0.2 μg/ml by Ogawa PM.
[c] Low-R, resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml by Ogawa PM.
[d] High-R, resistant to INH at 1.0 μg/ml by Ogawa PM.

ports (16, 17, 18, 26, 27, 30, 36, 40). Mutations in codon 315 of the *katG* gene are also found to be markers for multidrug-resistant *M. tuberculosis* and are successfully transmitted within the population (40). In our study, strains with *katG* codon 315 mutations showed comparably high frequencies of resistance to other drugs and multidrug resistance, 88.9% and 59.3%, respectively.

It seems that the mechanism of *M. tuberculosis* resistance to INH and ETH may involve an altered level of InhA, an expression which may have been influenced by the sequence change in the regulatory region of the *inhA* locus (7, 25, 27). Our present study showed that 43 (44.8%) out of 96 INH-resistant strains were also resistant to ETH. When ETH-resistant strains were divided into three groups based on the results of INH susceptibility testing with the Ogawa PM, 100% (11/11) of the strains in the Low-S group had mutations in the regulatory region of the *inhA* gene while only 5.9% (1/17) of the High-R strains carried mutations in this region. These findings suggest that for the low-level INH-resistant strains, mutations in the regulatory region of the *inhA* gene are associated with resistance to ETH but this is not true for the High-R strains (resistant to INH at 1.0 μg/ml). For the High-R strains, resistance to ETH may be conferred by another mechanism(s). Four out of 25 strains with *inhA* regulatory region mutations were susceptible to ETH, indicating that these mutations are not predicative of ETH resistance.

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